# Lack of chicken adaptation of newly emergent Eurasian H5N8 and reassortant H5N2 high pathogenicity avian influenza viruses in the U.S. is consistent with restricted poultry outbreaks in the Pacific flyway during 2014–2015

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## ARTICLE INFO

## ABSTRACT

In 2014–2015, the U.S. experienced an unprecedented outbreak of Eurasian clade 2.3.4.4 H5 highly pathogenic avian influenza (HPAI) virus, initially affecting mainly wild birds and few backyard and commercial poultry premises. To better model the outbreak, the pathogenesis and transmission dynamics of representative Eurasian H5N8 and reassortant H5N2 clade 2.3.4.4 HPAI viruses detected early in the North American outbreak were investigated in chickens. High mean chicken infectious doses and lack of seroconversion in survivors indicated the viruses were poorly chicken adapted. Pathobiological features were consistent with HPAI virus infection, although the delayed appearance of lesions, longer mean death times, and reduced replication in endothelial cells differed from features of most other Eurasian H5N1 HPAI viruses. Although these initial U.S. H5 HPAI viruses had reduced adaptation and transmissibility in chickens, multi-generational passage in poultry could generate poultry adapted viruses with higher infectivity and transmissibility.

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## 1. Introduction

Outbreaks of H5N8 Eurasian A/goose/Guangdong (Gs/GD) lineage clade 2.3.4.4 high pathogenicity avian influenza (HPAI) virus (World Organisation for Animal Health (OIE), 12 January 2015) were reported in South Korea during January 2014 in chickens and domestic ducks, and spread to other parts of Asia during that year (Jeong et al., 2014; Kim et al., 2014; Lee et al., 2014). By the end of 2014 this virus had spread intercontinentally, reaching Europe, in particular Germany, the Netherlands, the United Kingdom and Italy (World Organisation for Animal Health (OIE), 2015), as well as North America. The initial incursion of this viral lineage into North America was detected in November 2014

as a newly emergent H5N2 HPAI virus on a turkey farm in British Columbia, Canada. The virus was a reassortant containing five Eurasian avian influenza (AI) virus gene segments (including the H5 2.3.4.4 hemagglutinin) and three North American wild bird lineage AI virus gene segments (Ip et al., 2015; Torchetti et al., 2015; World Organisation for Animal Health (OIE), 2015). In December 2014 in Washington state of the U.S., an H5N8 HPAI virus containing all 8 gene segments of Eurasian origin and the reassortant H5N2 HPAI virus were detected in a captive-reared gyrfalcon (*Falco rusticolus*) and a wild Northern pintail duck (*Anas acuta*), respectively. Over the next 7 months, the U.S. experienced an unprecedented outbreak of H5 HPAI with detections of virus in wild waterfowl, wild and captive birds of prey, and backyard and commercial poultry flocks throughout the Northwestern and upper Midwestern states across the Pacific, Central, and Mississippi wild bird flyways (Jhung et al., 2015; United States Department of Agriculture, Animal and Plant Health Inspection Service, 2015). This HPAI outbreak represents the worst HPAI event for U.S. poultry producers, with more than 48 million birds died or culled during the control program (United States Department of

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**Table 1**
Experimental design and mortality of inoculated and contact 4-week-old chickens challenged with A/northern pintail/Washington/40964/2014 (H5N2) virus and A/gyrfalcon/Washington/40188-6/2014 (H5N8) virus, with mean chicken infectious and lethal doses.

| Challenge virus | Study/Dose | # of birds (inoculated+contact) | Mortality (MDT* expressed as dpc) | | CID$_{50}$ and CLD$_{50}$ (log$_{10}$) |
|---|---|---|---|---|---|
| | | | Inoculated | Contact | |
| H5N2 | CID$_{50}$+transmission $10^2$ EID$_{50}$ | 5+3 | 0/5 | 0/3 | 5.7 |
| | CID$_{50}$+transmission $10^4$ EID$_{50}$ | 5+3 | 0/5 | 0/3 | |
| | CID$_{50}$+transmission $10^6$ EID$_{50}$ | 5+3 | 3/5 (3) | 0/3 | |
| | Pathogenesis $10^6$ EID$_{50}$ | 10 | na | na | |
| H5N8 | CID$_{50}$+transmission $10^2$ EID$_{50}$ | 5+3 | 0/5 | 0/3 | 4.4 |
| | CID$_{50}$+transmission $10^4$ EID$_{50}$ | 5+3 | 2/5 (4) | 0/3 | |
| | CID$_{50}$+transmission $10^6$ EID$_{50}$ | 5+3 | 5/5 (4.1) | 0/3 | |
| | Pathogenesis $10^6$ EID$_{50}$ | 10 | na | na | |
| – | sham | 4 | 0/4 | 0/4 | – |

CID50, mean chicken infectious dose; CLD50, mean chicken lethal dose; dpc, days post-challenge; EID50, mean egg infectious dose; H5N2, A/northern pintail/WA/40964/2014; H5N8, A/gyrfalcon/WA/40188-6/2014; MDT, mean death time; na, not applicable.

* #dead birds × dpc/total dead birds.

Agriculture, Animal and Plant Health Inspection Service, 2015) and bans on imports of U.S. poultry and poultry products from many different countries (United States Department of Agriculture, Food Safety and Inspection Service, 2015).

The introduction and rapid spread of these H5 HPAI viruses from Asia into Europe and North America likely involved movement of the virus by migratory waterfowl (Adlhoch et al., 2014; Avian influenza, 2015). These H5 HPAI viruses first appeared and spread within the Pacific flyway during the winter of 2014–2015 (Jhung et al., 2015; United States Department of Agriculture, Animal and Plant Health Inspection Service, 2015) with the majority of detections in wild waterfowl (n=70) and birds of prey (n=4), and a few poultry cases (n=10), mostly in mixed backyard poultry with outdoor exposure (n=8), and rarely in indoor commercial poultry (n=2) (Jhung et al., 2015; United States Department of Agriculture, Animal and Plant Health Inspection Service, 2015). The epidemiology of the reported detections indicated that, on the one hand, good biosecurity practices likely prevented outbreaks in indoor commercial operations along the Pacific flyway. On the other hand, it suggested that the initial H5N2 and H5N8 HPAI viruses detected in the U.S. were highly adapted to waterfowl and not yet well adapted to domestic poultry. Taking into account these combined factors, the viruses caused low mortality rates in wild birds but could still replicate to high titers (Kang et al., 2015), enabling wild waterfowl to survive long migrations and spread the virus to new exposed populations, such as outdoor reared poultry. In contrast, these H5 clade 2.3.4.4 viruses are highly pathogenic for gallinaceous poultry and at least some species of raptors (United States Department of Agriculture, Animal and Plant Health Inspection Service, 2015), but there is still a lack of knowledge concerning the infection dynamics, transmission, and virulence of the North American viruses for chickens and any differences in such biological characteristics between the wholly Eurasian H5N8 and the reassortant H5N2 viruses. In the present study, the pathogenesis and transmission dynamics of initial H5N2 and H5N8 clade 2.3.4.4 HPAI viruses detected in the North American outbreak were investigated in White Leghorn (WL) chickens.

## 2. Materials and methods

### 2.1. Viruses

The influenza A isolates A/gyrfalcon/Washington/40188-6/2014 (H5N8) and A/northern pintail/Washington/40964/2014 (H5N2) were used as challenge viruses. These were the first two HPAI isolates from the U.S. outbreak and they are considered representative of both the wholly Eurasian H5N8 lineage viruses and reassortant Eurasian/North American lineage H5N2 viruses, respectively. The viruses were propagated and titrated by allantoic sac inoculation of 9–10 day-old embryonating chicken eggs (ECE) by standard methods (Swayne et al., 1998).

### 2.2. Animals and housing

Specific pathogen free (SPF) WL chickens (*Gallus domesticus*; Southeast Poultry Research Laboratory (SEPRL), Athens, GA) were utilized. Prior to inoculation, a representative number of chickens were tested and shown to be serologically negative for AI virus infection as determined by hemagglutinin inhibition (HI) test. Also, oral and cloacal swabs were collected to ensure absence of virus shedding as determined by quantitative real-time RT-PCR (qRRT-PCR). Each experimental group was housed separately in negative pressure isolators with HEPA-filtered ventilation within the animal biosafety level 3 enhanced facilities at SEPRL. The birds had *ad libitum* access to feed and water. All procedures were performed according to the requirements of protocols approved by the Institutional Animal Care and Use Committee, and Institutional Biosecurity Committee.

### 2.3. Experimental design and sampling

#### 2.3.1. Infectivity and transmission study

To evaluate the mean chicken infectious dose (CID$_{50}$) and the mean chicken lethal dose (CLD$_{50}$) of each isolate, 4-week-old chickens were divided into three groups (n=5/group), each inoculated intranasally with $10^2$, $10^4$, or $10^6$ EID$_{50}$/0.1 ml of respective viruses (Table 1). Sham birds were inoculated intranasally with 0.1 ml of sterile allantoic fluid diluted 1:300 in brain heart infusion (BHI) media (Becton, Dickinson and Company, Sparks, MD). The inoculum titers were subsequently verified by back titration in ECE as $10^{2.7}$, $10^{4.5}$, and $10^{7.1}$ EID$_{50}$/0.1 ml for the H5N2 virus and $10^{2.6}$, $10^{4.7}$, and $10^{6.3}$ EID$_{50}$/0.1 ml for the H5N8 virus. To evaluate the transmissibility of each isolate, three non-inoculated hatch-mates were added to each dose group at 1 day post-challenge (dpc). Clinical signs were monitored twice a day during the first 4 dpc and daily thereafter. Oral swabs were collected from all the birds daily for the first 5 dpc, placed in 1.5 ml of BHI with penicillin (2000 units/ml; Sigma Aldrich), gentamicin (200 ug/ml; Sigma Aldrich) and amphotericin B (5 ug/ml; Sigma Aldrich), and stored at −80 °C. Severely sick birds were euthanized and counted as dead for the next day. At 14 dpc, surviving birds were bled to

evaluate antibody titers and euthanized by cervical dislocation.

### 2.3.2. Pathogenesis study

Four-week-old chickens were inoculated intranasally with $10^6$ $EID_{50}/0.1$ ml of either H5N2 or H5N8 viruses (Table 1). Sham inoculated birds were inoculated intranasally with 0.1 ml of sterile allantoic fluid diluted 1:300 in BHI with antibiotics. The inoculum titers were subsequently verified by back titration in ECE as $10^{6.1}$ and $10^{6.5}$ $EID_{50}/0.1$ ml for H5N2 HPAI and H5N8 HPAI viruses, respectively. Clinical signs were monitored twice a day during the first 4 dpc and daily thereafter. For each virus, two birds were necropsied at five time-points based on clinical progression: asymptomatic (twice: 18 and 24 hours post-challenge (hpc)), listless, severely lethargic, and dead. One sham-inoculated bird was euthanized and necropsied at the first and the last necropsy time-points. Necropsies and tissue sampling were performed according to a standard protocol (Majo and Dolz, 2011). Portions of nasal cavity, brain, thymus, trachea, lung, proventriculum, duodenum, pancreas, jejunum-ileum, spleen, kidney, adrenal and gonad, liver, skeletal muscle, comb, and heart were collected in 10% buffered formalin (Thermo Fisher Scientific, Waltham, MA) for histopathologic evaluation. Brain, spleen, heart, and lung were also collected in BHI with antibiotics to a 10% (wt/vol) concentration for viral RNA quantification by qRRT-PCR and stored at $-80\,°C$. Severely sick birds were euthanized and counted as dead for the next day. At 10 dpc, surviving birds were bled to evaluate antibody titers and euthanized by cervical dislocation.

### 2.4. Viral RNA quantification in swabs and tissues

Swabs and tissues in BHI were processed for qRRT-PCR to determine viral RNA titers. Viral RNA was extracted using Mag-MAX™-96 AI/ND Viral RNA Isolation Kit® (Ambion, Inc.) following the manufacturer's instructions. In tissue homogenates, and in order to standardize the amount of nonspecific RNA from the tissue, the resulting viral RNA extracts were quantified by Nano-Drop™ 1000 Spectrophotometer (Thermo Fisher Scientific) following the manufacturer's instructions and accordingly diluted with phosphate buffered saline (PBS) to obtain 50 ng/µl. The resulting viral RNA extracts, diluted (tissue homogenates) or undiluted (swabs), were quantified by one-step qRRT-PCR which targets the influenza matrix gene (Spackman et al., 2002) using 7500 FAST Real-time PCR System (Applied Biosystems, Foster City, CA, USA) or the Smartcycler 2 (Cepheid Corp, Sunnyvale, CA), and the AgPath-ID OneStep RT-PCR kit (Ambion, Inc.). The standard curves for viral RNA quantification were established with RNA extracted from dilutions of the same titrated stocks of the challenge viruses. The limit of detection was $10^{1.7}$ $EID_{50}/ml$ for H5N2 virus and $10^{1.9}$ $EID_{50}/ml$ for H5N8 virus; therefore for statistical purposes H5N2 qRRT-PCR negative samples were given a numeric value of $10^{1.6}$ $EID_{50}/ml$, and H5N8 qRRT-PCR negative samples were given a numeric value of $10^{1.8}$ $EID_{50}/ml$.

### 2.5. Statistical analysis

The D'Agostino and Pearson test was used to assess the normality of distribution of investigated parameters. All parameters in our study were not normally distributed. Significant difference for mean viral titers in tissues between groups was analyzed using Mann-Whitney test (GraphPad Prism™ Version 5 software). A P-value of $<0.05$ was considered to be significant.

### 2.6. Histopathology and immunohistochemistry

Tissues in formalin were processed for routine hematoxylin/eosin staining. Tissues were also processed for immunohistochemical staining as previously described (Perkins and Swayne, 2001) with minor modifications, using a mouse-derived monoclonal antibody (P13C11, developed at SEPRL) specific for type A influenza virus nucleoprotein.

### 2.7. Serology

Sera samples were tested by HI assays against antigens specific for the challenge viruses. The antigens were prepared as previously described (Abbas et al., 2011) and the HI assays were performed according to standard procedures (Pedersen, 2008). Titers were calculated as the reciprocal of the last HI positive serum dilution and samples with HI titers of 8 ($2^3$) or below were considered negative.

## 3. Results

### 3.1. Infectivity and transmission study

Quantitation of viral shedding was performed by qRRT-PCR using extrapolation of a standard curve generated with each virus. Because the birds at each dose were co-housed in these studies, there is the possibility of detection of viral RNA from samples because the birds share common watering cups, food troughs and are in close contact with other potentially infected birds, but not necessarily because of actual virus replication and shedding. Therefore, birds were considered infected if they had detectable virus along with clinical disease, mortality, or if they seroconverted using the HI tests at 14 dpc. Only 60% of the chickens inoculated with the high dose of H5N2 virus became infected and died with a mean death time (MDT) of 3 dpc (Table 1), resulting in $10^{5.7}$ $CID_{50}$. None, 40%, and 100% of the chickens with the low, medium, and high dose of H5N8 virus, respectively, became infected and died with a MDT of 4 dpc (Table 1), resulting in $10^{4.4}$ $CID_{50}$. The surviving birds did not show evidence of clinical disease and they were all serologically negative based on HI data and all were considered as uninfected. Therefore, the $CID_{50}$ and the $CLD_{50}$ for both viruses were the same in this study. No contact birds became infected based on morbidity, mortality (Table 1), or serology.

Birds inoculated with the low and medium doses of H5N2 virus did not shed detectable levels of virus (Fig. 1A and B) and were all seronegative at 14 dpc thus were considered uninfected. In contrast, birds inoculated with the highest dose of H5N2 virus either shed high amounts of virus from 1 to 4 dpc and subsequently died, or shed no detectable virus for the course of the experiment and were seronegative at termination (Fig. 1C). In particular, 3 birds shed virus the first 3 dpc and 2 birds shed virus at 4 dpc; 3 dpc was the peak day of shed with a mean titer of the positive birds of 7.2 $log_{10}$ $EID_{50}/ml$ (Fig. 1C). Birds inoculated with the low dose of H5N8 virus did not shed detectable virus, similar to the H5N2 low dose group (Fig. 1D). However, birds inoculated with the medium and highest dose of H5N8 virus either shed high amounts of virus before dying, or shed no/minimal detectable virus for the course of the experiment and were seronegative at termination (Fig. 1E and F).

The chicken transmission studies were conducted by introducing 3 naïve contact transmission birds into the isolators 24 h after the direct intranasal inoculation of 5 chickens. Virus was only sporadically detected in oral swabs of contact transmission birds at 2, 3, and 4 days post-exposure (dpe) with no evidence of clinical disease, mortality, or seroconversion (data not shown). The sporadic positive samples were primarily from the highest dose groups and were considered environmental contamination. Therefore, virus transmission by direct contact was determined to have been unlikely.
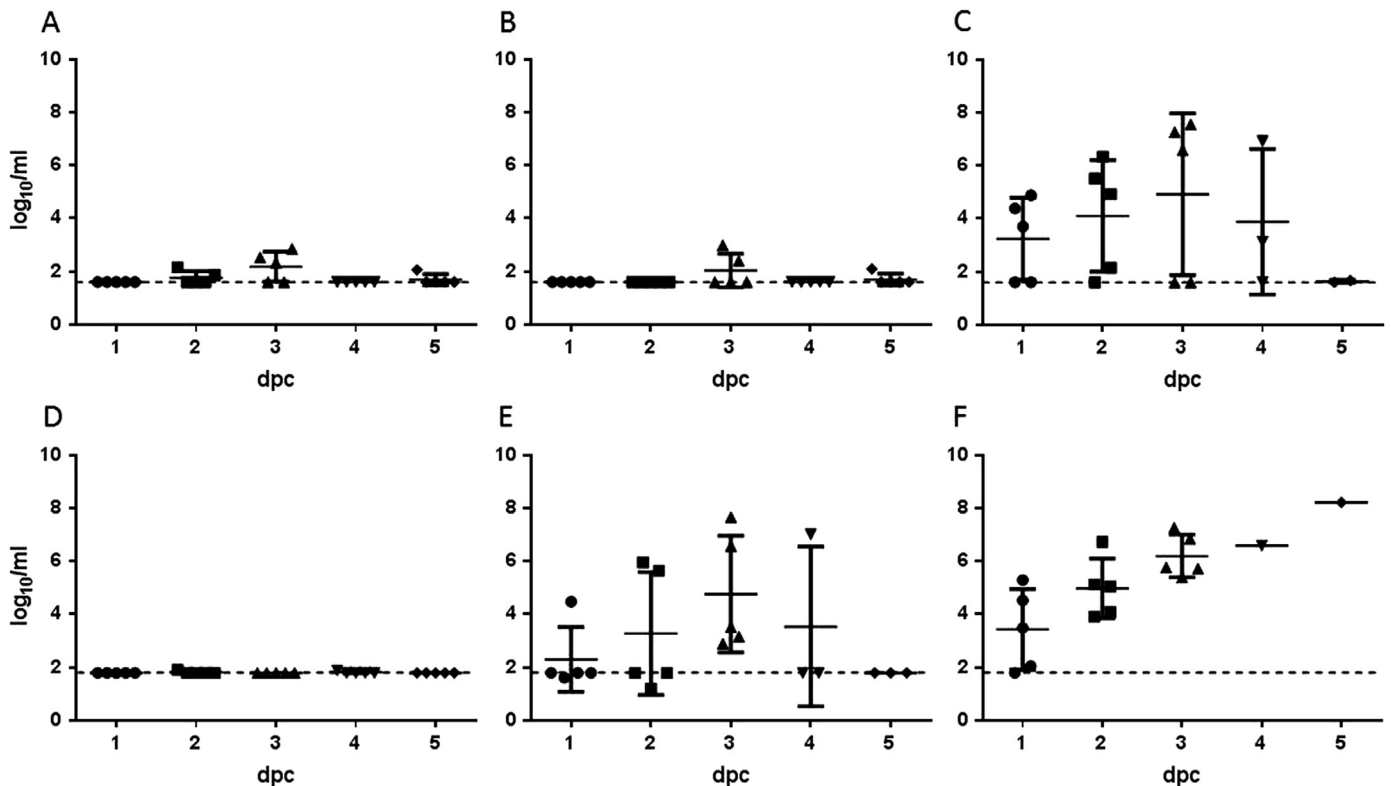
**Fig. 1.** Mean viral oral shed detected by qRRT-PCR from 4-week-old chickens directly inoculated with A/northern pintail/Washington/40964/2014 (H5N2) virus and A/gyrfalcon/Washington/40188-6/2014 (H5N8) virus by dose. A. $10^{2.7}$ $EID_{50}$/0.1 ml of H5N2 virus per bird. B. $10^{4.5}$ $EID_{50}$/0.1 ml of H5N2 virus per bird. **C.** $10^{7.1}$ $EID_{50}$/0.1 ml of H5N2 virus per bird. D. $10^{2.6}$ $EID_{50}$/0.1 ml of H5N8 virus per bird. E. $10^{4.7}$ $EID_{50}$/0.1 ml of H5N8 virus per bird. F. $10^{6.3}$ $EID_{50}$/0.1 ml of H5N8 virus per bird. The limit of detection was $10^{1.7}$ $EID_{50}$/ml for H5N2 virus and $10^{1.9}$ $EID_{50}$/ml for H5N8 virus; therefore a numeric value of $10^{1.6}$ $EID_{50}$/ml and $10^{1.8}$ $EID_{50}$/ml was used for H5N2 and H5N8 qRRT-PCR negative samples, respectively.

## 3.2. Pathogenesis study

Mild illness was evident by 42 hpc, consisting of nonspecific clinical signs such as ruffled feathers, listlessness, and infraorbital swelling with both viruses. Severe illness including neurological signs and prostration was observed by 4 dpc in 2 birds inoculated with the H5N8 virus and by 4.5 dpc in one bird inoculated with H5N2 virus.

Similar patterns of severity and timeline of gross lesions were observed in H5N2 and H5N8 virus inoculated chickens. No gross lesions were observed in asymptomatic birds necropsied at 18 and 24 hpc. In listless birds (all necropsied on 42 hpc), the most common lesion was multifocal necrosis in the pancreas (Fig. 2A), sometimes accompanied by hemorrhagic duodenum. Enlarged kidneys, congested lungs, splenomegaly with parenchymal mottling, and enlarged thymus were also observed in almost all the listless birds. One bird inoculated with H5N8 virus also had edematous fluid in the lung. No H5N2-inoculated severely lethargic birds were observed and thus none were available for post mortem examination. By contrast, on 3 dpc H5N8-inoculated severely lethargic birds had cyanotic combs (Fig. 2B) and wattles, and hemorrhages on the shanks. Parenchymal mottling was observed on the spleen and the proventriculi, as well as necrotic and hemorrhagic duodenum and pancreas. One H5N8-inoculated severely lethargic bird also had congested lungs. Dead birds inoculated with H5N2 virus were necropsied on 3 and 4.5 dpc, and they had cyanotic combs and wattles, splenomegaly with parenchymal mottling, parenchymal pallor on the kidneys, fibrinous and necrotic myocardium with petechial hemorrhages (Fig. 2C), and necrotic pancreas. Dead birds inoculated with H5N8 virus were necropsied on 3 and 5.5 dpc, with very similar lesions to dead H5N2-inoculated birds.

Similar histological lesions, severity of lesions and time of lesion appearance were observed for H5N2 and H5N8 virus inoculated chickens. Lesions or viral antigen were first observed at 42 hpc and included multifocal necrosis of nasal epithelium (Fig. 2D), mild splenic necrosis, thymic lymphocyte depletion, and multifocal necrosis of dermis of the comb and head (Fig. 2E) with viral antigen observed in splenic periarteriolar sheath support cells, scattered hepatic Kupffer cells, clusters of cardiac myocytes, nasal submucosal capillary endothelial cells and associated macrophages, and capillary endothelial cells in comb submucosa associated with areas of necrosis and within epidermal cells. In birds that died (4 to 5.5 dpc), multifocal necrosis with viral antigen was widespread in the parenchymal cells of most tissues, especially prominent in brain, heart, adrenal gland, and comb (Fig. 2F–L). Widespread viral antigen staining in capillary endothelial cells was lacking, with such staining restricted mainly to capillaries in the dermis of comb and air capillaries of the lungs.

Brain, spleen, heart, and lung were collected from each necropsied bird in BHI with antibiotics for viral RNA quantification by qRRT-PCR. Mean virus titers per tissue and time point were calculated using the 2 birds from each virus challenge necropsied at each time point. For each stage of the clinical progression, virus titers were statistically similar when comparing H5N2 and H5N8 inoculated groups (Fig. 3). Asymptomatic infected birds necropsied at 18 and 24 hpc already presented detectable challenge virus in most of the tissues, ranging from 1.7 $\log_{10}$ $EID_{50}$/ml of spleen (H5N2 virus) to 3.0 $\log_{10}$ $EID_{50}$/ml of heart (H5N8 virus). Tissues from listless infected birds necropsied at 42 hpc presented significantly higher virus titers compared to asymptomatic birds, ranging from 2.7 $\log_{10}$ $EID_{50}$/ml of spleen (H5N8 virus) to 4.6 $\log_{10}$ $EID_{50}$/ml of heart (H5N2 virus). Virus titers in tissues of morbid
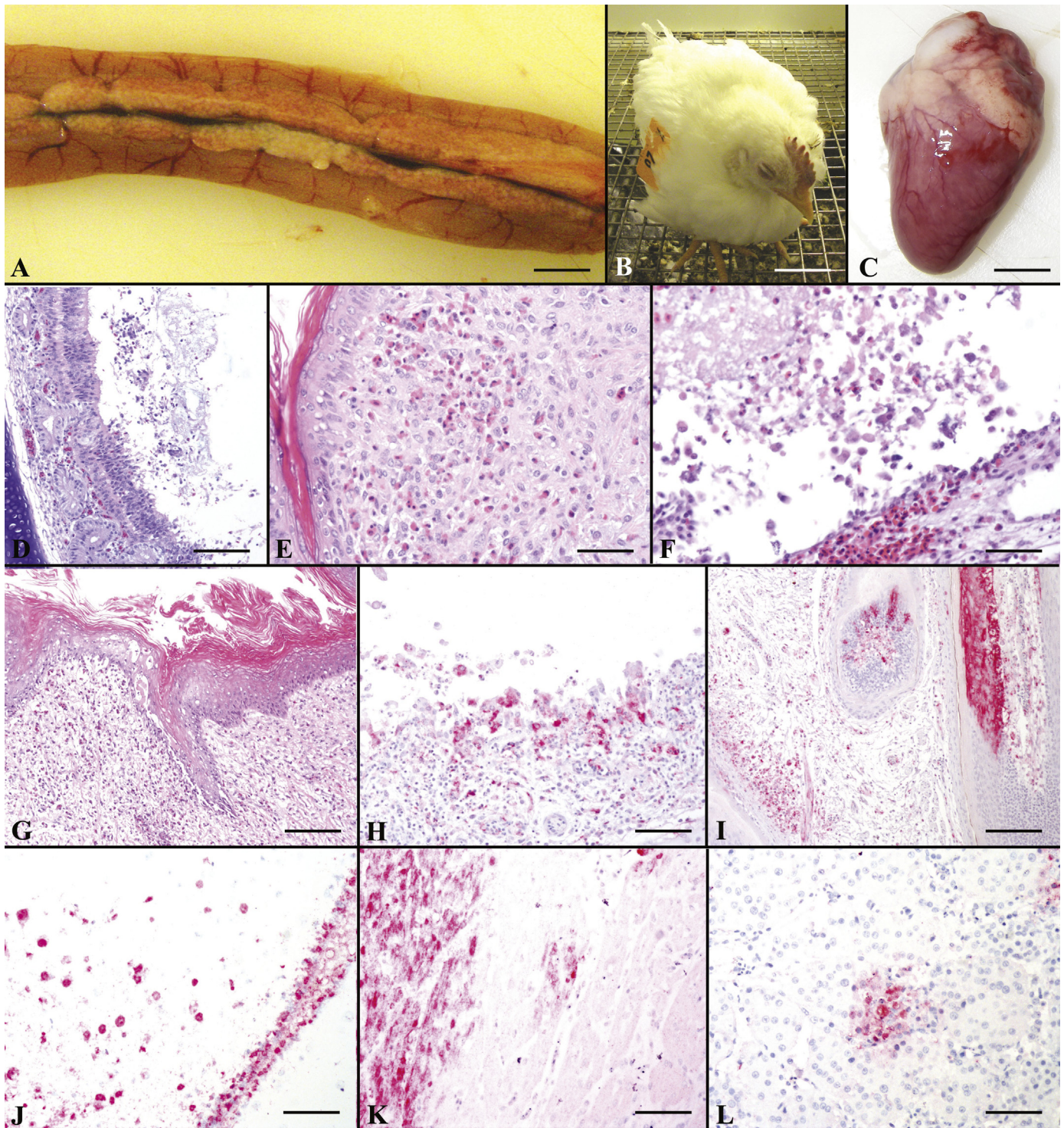
**Fig. 2.** Gross (A–C) and histological (D–G) lesions and immunohistochemical detection of viral antigen (H-L) in 4-week-old chickens intranasally inoculated with $10^{6.1}$ $EID_{50}$ /0.1 ml of A/northern pintail/Washington/40964/2014 (H5N2) virus or $10^{6.5}$ $EID_{50}$/0.1 ml of A/gyrfalcon/Washington/40188-6/2014 (H5N8) virus. A. Multifocal coalescing areas of pancreatic necrosis, H5N8 virus, 5.5 dpc, bar=5 mm. B. Listless chicken with necrotic tips on the comb, H5N8 virus, 4.5 dpc, bar =40 mm. C. Multiple hemorrhages on epicardium, H5N2 virus, 4.5 dpc, bar=7 mm. D. Mild rhinitis with luminal mucus, exudate epithelium and some inflammatory cells, H5N2 virus, 42 hpi, bar=144 mm. E. Heterophilic dermatitis with early vacuolar degeneration of basilar epithelium of the comb, H5N8 virus, 42 hpc, bar=144 μm. F. Necrosis of nasal epithelium with lumen debris and heterophils from site of inoculation, H5N2 virus, 4 dpc, bar=144 μm. G. Severe necrotizing epidermitis and edematous dermatitis of the comb, H5N2 virus, 4 dpc, bar=72 μm. H. Viral antigen (in red) in nasal epithelium and cellular debris, H5N2 virus, 4 dpc, bar = 144 μm. I. Viral antigen in feather follicle epithelium, erector pili smooth muscle and perifollicular cells, H5N2 virus, 4 dpc, bar=72 μm. J. Viral antigen in cortical neurons and ependymal cells, H5N8 virus, 4 dpc, bar=144 μm. K. Viral antigen in cardiac myocytes, H5N8 virus, 4 dpc, bar=144 μm. L. Viral antigen in adrenal corticotropic cells, H5N8 virus, 4 dpc, bar=144 μm.

and dead birds necropsied at 3 dpc or later were significantly higher than any other time point, with peak levels in heart (7.7 $\log_{10}$ $EID_{50}$/ml) and brain (8.6 $\log_{10}$ $EID_{50}$/ml).

All the birds that survived were seronegative at termination indicating they likely were not infected with the inoculated dose of virus. Sham-inoculated birds were clinically healthy throughout the experiment, without observation of lesions and a lack of antigen detection.
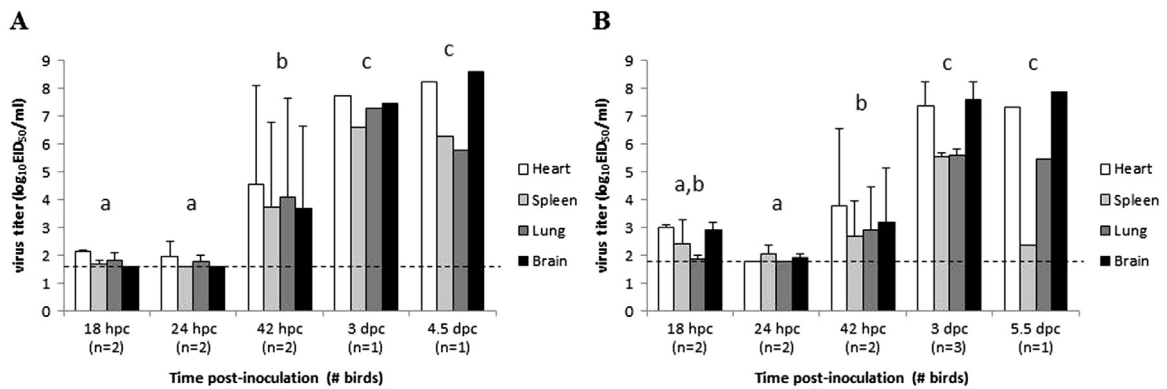
**Fig. 3.** Virus detection by qRRT-PCR in tissues of 4-week-old chickens directly inoculated with A. $10^{6.1}$ EID$_{50}$/0.1 ml of A/northern pintail/Washington/40964/2014 (H5N2) virus per bird and B. $10^{6.5}$/0.1 ml EID$_{50}$ of A/gyrfalcon/Washington/40188-6/2014 (H5N8) virus per bird. The limit of detection was $10^{1.7}$ EID$_{50}$/ml for H5N2 virus and $10^{1.9}$ EID$_{50}$/ml for H5N8 virus; therefore a numeric value of $10^{1.6}$ EID$_{50}$/ml and $10^{1.8}$ EID$_{50}$/ml was used with H5N2 and H5N8 qRRT-PCR negative samples, respectively.

## 4. Discussion

In this chicken study, we investigated the pathogenesis and transmission dynamics of initial H5N2 and H5N8 clade 2.3.4.4 HPAI viruses detected in the 2014 North American outbreak (Jhung et al., 2015; United States Department of Agriculture, Animal and Plant Health Inspection Service, 2015) to better understand the unexpected high number of wild bird detections and the low number of affected backyard and commercial poultry premises across a wide geographic area in the Western U.S. All the parameters assessed in the present study indicated the viruses that were tested, which originated from a wild duck and a captive gyrfalcon, were poorly adapted to chickens, based on the high CID$_{50}$ of $10^{5.7}$ EID$_{50}$ for H5N2 virus and $10^{4.4}$ EID$_{50}$ for H5N8 virus. Previously, the CID$_{50}$ was determined for a number of other HPAI viruses, and that value was proposed as a predictor of infectivity, adaptation, and transmission and maintenance to a specific poultry host (Swayne and Slemons, 2008). In particular, our CID$_{50}$ were 3.3 and 2 logs higher, respectively, than the Asian A/Hong Kong/486/1997 (H5N1) virus (CID$_{50}$ of $10^{2.4}$ EID$_{50}$) (Swayne and Slemons, 2008). This virus belongs to the Eurasian Gs/GD H5 HPAI clade 0 and is an ancestor of the viruses used in this study (Ip et al., 2015), known to be one of the most virulent and poultry-adapted HPAI viruses (Perkins and Swayne, 2001; Spickler et al., 2008). The experimental CID$_{50}$ of $10^{4.7}$ EID$_{50}$ was observed as the upper cutoff for viruses to be sufficiently adapted to chickens to be transmissible in the field and produce one or more affected premises, although large sustained outbreaks have been caused by viruses with a CID$_{50} \leq 10^{3.1}$ EID$_{50}$ (Swayne and Slemons, 2008). In the current study, the CID$_{50}$ of the viruses are close to (H5N8 virus) or above (H5N2 virus) the upper cut-off value, suggesting a lack of adaptation to poultry. Host adaptation, together with other epidemiological factors such as flock density or effective biosecurity, would account for reduced transmission to chickens and other gallinaceous poultry with only a few affected poultry premises within the Pacific flyway. This was further corroborated in the current study by the poor virus transmission to naïve chickens that were directly exposed to inoculated chickens even though some or all the inoculated birds in the high challenge groups were shedding high levels of virus at early time points within the study.

In reference to virus replication and shedding of the HPAI viruses, the quantity of virus shed has a direct impact on the degree of environmental contamination and subsequent bird-to-bird transmission and ultimately farm-to-farm spread. Some or all the inoculated birds in the medium (for H5N8 virus) and high (for both viruses) inoculated groups were shedding high levels of virus before dying at 4–5 dpc. Experimental contact-transmission was not observed based on clinical signs, mortality or seroconversion.

Thus, the sporadic oral detection of virus in a few contact birds, primarily in the highest dose groups, suggests detection of environmental contamination from coprophagous behavior without replication in the exposed host chicken. It is possible that limited replication after either direct inoculation of the virus or by environmental exposure occurred with some birds, but the innate immune response was effective in clearing the virus before it could cause clinical disease or present enough antigen to the immune system to stimulate an immune response. Therefore, although limited replication is possible, it seems unlikely that the low amounts of virus detected would contribute to the infection of other birds. The overall virus shedding results suggest low potential of these viruses to transmit and spread within the chicken host population (Swayne and Slemons, 2008), unlike their Eurasian Gs/GD H5N1 HPAI virus ancestors (Kim et al., 2014; Perkins and Swayne, 2001). However, both viruses replicated systemically in infected chickens when inoculated at a high dose, even among asymptomatic birds to at least 42 h, with significantly higher titers among clinically ill birds. Interestingly, virus loads in tissues corresponded to clinical progression of infection and time of death; i.e., the presence of virus in the brain was low in asymptomatic birds and reached very high titers by 3 dpc, corresponding to observation of neurological signs at 4 dpc.

The early detection of HPAI virus is the key to rapid control and eradication in the U.S. and many other countries. In the current study, the clinical lesions observed with both the H5N2 and H5N8 viruses were consistent with lesions expected with a HPAI virus, including hemorrhages in the legs, comb, wattle, and petechial hemorrhages and necrosis in several different organs. Neurologic signs, although not a common clinical sign, provide evidence of infection in the brain which should make clinicians consider HPAI in the differential diagnosis. Uncharacteristically of these viruses is the longer incubation period compared to typical Gs/GD lineage H5N1 HPAI viruses, which in experimental conditions often kill challenged birds within 48 h (Perkins and Swayne, 2001; Spickler et al., 2008; Swayne, 2000). The Gs/GD lineage viruses usually produce infection and accompanying inflammatory and necrotic lesions in multiple visceral organs, the brain and skin, as was seen with infections by the current high challenge dose of both H5 HPAI viruses (Perkins and Swayne, 2001; Spickler et al., 2008; Swayne, 2000). However, the delayed appearance of lesions and MDT for the viruses during the current study was over 4 days. Longer survival could possibly be related to reduced replication in vascular endothelial cells and more extensive replication in parenchymal cells of visceral organs (Nicholls et al., 2007). Longer incubation periods could present a significant challenge to AI virus monitoring efforts in domestic farms if such monitoring predominantly relies on acute clinical signs and rapid appearance of

high mortality rates, which is expected with other HPAI viruses (Spickler et al., 2008). Therefore, introducing additional parameters as triggers for AI sampling and testing could be critical for early virus detection, such as drops in water or feed consumption and, with birds in lay, a drop in egg production. Unrecognized virus shedding does not only enable virus to spread within a farm or among farms, but it also facilitates the continuous circulation in birds required to accumulate mutations essential to alter host range, replication efficiency, virulence, and/or transmission in appropriate hosts, in this case the poultry population (Li and Cardona, 2010).

The subsequent outbreak of H5N2 in the Midwest, which affected over 48 million birds with strong evidence of farm to farm transmission, seems at odds with the data presented. Our standard transmission studies used SPF chickens in isolators that maintained constant environmental conditions. Such controlled laboratory setting provides the best opportunity for comparison between different viruses (Swayne and Pantin-Jackwood, 2006). However, conditions in the field are considerably different than experimental conditions, and birds in the field are likely more susceptible to infection due to stress of egg production, higher density of birds, concomitant infections, or immunosuppression, as common adverse environmental conditions. Therefore, field conditions likely contributed to the outbreaks that were observed in the Midwest, and probably facilitated spread. The virus also likely adapted to chickens and turkeys during the Midwestern outbreak increasing infectivity and transmission, and facilitating spread. In addition to the virus infectivity for chickens, other epidemiological factors (e.g., weather, routine biosecurity practices, flock density and composition, etc.) may have also contributed to the differences in reported infections and virus spread between the Midwest and the Pacific regions.

Our study concludes that the early wild bird Eurasian H5N8 HPAI and reassortant H5N2 viruses from the initial cases within the Pacific flyway were not yet optimally adapted to chickens based on the current experimental findings of high CID$_{50}$, longer MDT, lack of transmission to contact birds, and limited blood vessel endothelial cell replication. However, these clade 2.3.4.4 H5 HPAI viruses continued to circulate in upper Midwestern U.S. in early 2015 with detections mostly in backyard and commercial poultry, and reduced detections in wild waterfowl (United States Department of Agriculture, Animal and Plant Health Inspection Service, 2015), suggesting increased adaptation to poultry. Further studies assessing transmission and pathogenesis of these two viruses in other gallinaceous species, as well as studies with more recent isolates from upper Midwestern U.S., are crucial for better understanding the evolution and poultry adaptation of Gs/GD lineage clade 2.3.4.4 H5 HPAI viruses.

## Conflict of interest

The authors declare they have no conflict of interest.

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